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RESEARCH ARTICLE | OCTOBER 15 2000

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J Immunol (2000) 165 (8): 4226–4230.

<https://doi.org/10.4049/jimmunol.165.8.4226>

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The Effects of Prolonged Administration of 5-Bromodeoxyuridine on Cells of the Immune System¹

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We have determined the *in vivo* effect of 5-bromodeoxyuridine (BrdU) administered to mice in the drinking water for various lengths of time on the performance of T and B lymphocytes in a number of experimental protocols. Young mice continuously exposed to BrdU fail to gain weight, and the lymphocytes recovered after a prolonged period of exposure are fewer in number than in control mice. The recovery of normal levels of T and B lymphocytes after irradiation is severely impaired. Ag-specific cells responding to Ag in an adoptive transfer model fail to expand as much in the presence of BrdU as in the absence, and the Ag-specific effectors produced in the presence of BrdU are less able to secrete cytokines upon restimulation *in vitro*. Polarized populations of Tc1 and Tc2 effectors generated *in vitro* proliferate less in the presence of BrdU, and the resulting effectors make less cytokines per cell upon restimulation. Thus, the incorporation of BrdU into T or B lymphocytes can, under some circumstances, seriously impair the performance of the labeled cells, and these findings raise a note of caution in the interpretation of studies that make use of long-term exposure to BrdU. *The Journal of Immunology*, 2000, 165: 4226–4230.

The incorporation of 5-bromodeoxyuridine (BrdU)⁴ into proliferating cells has been used as a measure of cell proliferation in a large number of studies of lymphocyte homeostasis and Ag-driven expansion, and it has been widely assumed that the incorporation of BrdU did not adversely affect the performance of the proliferating cells. The main purpose of this study is to determine under what circumstances this assumption may be in error rather than to determine the mechanism of the deleterious effects.

Thymidine and its analogues are taken up into the cell by a process of facilitated diffusion under the influence of a permease enzyme and are phosphorylated to deoxynucleotide monophosphate by the salvage pathway enzyme, thymidine kinase (1). The BrdU monophosphate derived from the salvage pathway competes with thymidine (5-methyldeoxyuridine) monophosphate of the *de novo* pathway derived from the deamination and methylation of cytidine deoxynucleoside monophosphate. The levels of some enzymes in the *de novo* pathway may vary and are under regulatory control. Levels of salvage enzymes can also vary. Thus, different cell lineages and developmental stages may have substantial differences in the relative amounts of the enzymes involved in the *de novo* and salvage pathways and may vary quite substantially in the efficiency of BrdU labeling (2, 3). This is generally ignored by immunologists, however, and it is assumed that the amount of proliferation is proportional to the amount of BrdU incorporated.

Another caveat is that continuous exposure to BrdU may select for a subset of cells with lower levels of the salvage enzymes or

higher levels of *de novo* pathway enzymes (4). Thus, function in the presence of BrdU may appear to be retained or recover, but could be the property of BrdU-resistant cells that can no longer label with BrdU.

A third caveat is that BrdU may alter the metabolism of the cell into which it is incorporated. This is of little consequence if the assay of BrdU incorporation is conducted immediately after incorporation and is merely used to determine the number of cells in the S phase of the cell cycle. In some studies, however, the BrdU-labeled cells are followed for considerable lengths of time. If the experiment calls for a pulse protocol, the BrdU may be diluted by subsequent division, and the deleterious effects of BrdU may be ameliorated. In other protocols, BrdU is made available continuously and may begin to seriously affect the behavior of the BrdU-labeled cells (5–12).

There is a very large literature on the nature of these effects, which range from death of the mouse, death of the BrdU-labeled cells, induction or inhibition of development in BrdU-labeled cells *in vitro*, and in changes in the expression of cell surface molecules (for a brief sampling, see Refs. 13–20). Other studies, however, have appeared to show that the incorporation of BrdU has no effect on lymphocyte homeostasis or function, and the worrying studies are largely ignored by immunologists.

In the experiments described in this work, we have measured a number of parameters of the performance of cells variously labeled with BrdU in different experimental models. It is clear from our results that the incorporation of BrdU can, under some circumstances, seriously impair the performance of the labeled cells and raise a note of caution in the interpretation of studies that make use of long-term exposure to BrdU.

Young mice continuously exposed to BrdU fail to gain weight, and the lymphocytes recovered after a prolonged period of exposure are fewer in number than in control mice. The recovery of normal levels of T and B lymphocytes after irradiation is severely impaired. Ag-specific cells responding to Ag in an adoptive transfer model fail to expand as much in the presence of BrdU as in the absence, and the Ag-specific effectors produced in the presence of BrdU are less able to secrete cytokines upon restimulation *in vitro*. Polarized populations of Tc1 and Tc2 effectors generated *in vitro*

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Received for publication March 28, 2000. Accepted for publication July 20, 2000.

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¹ This work was supported, in part, by a grant from the National Institutes of Health.

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⁴ Abbreviation used in this paper: BrdU, 5-bromodeoxyuridine.

proliferate less in the presence of BrdU, and the resulting effectors make less cytokines per cell upon restimulation.

Materials and Methods

Mice

Male or female C56BL/6 mice were used at 6 wk of age, unless otherwise specified. In some experiments, OT-1 mice (C57BL/6 mice bearing the α - and β -chains of the TCR specific for the OVA peptide SIINFEKL presented on K^b) were used (21).

Antigens

OVA was obtained from Sigma (St. Louis, MO).

Flow cytometry

The following mAbs were used for immunofluorescent staining: Cy-chrome anti-CD8 (PharMingen, San Diego, CA), FITC anti-CD44 (PharMingen; clone IM7), FITC anti-CD4 (GK1.5), PE anti-mouse CD19 (PharMingen, 1D3), and FITC anti-BrdU (Becton Dickinson, San Jose, CA), used as described in the package insert. After staining with the appropriate mAbs, FACS analysis was conducted on a FACScan (Becton Dickinson) by using the CellQuest software. All plots shown were gated on the propidium iodide-negative, live population.

Irradiation

Mice received whole body irradiation in an AECL Gammacell 40 irradiator, at rate of 62 Gy/h, for a total dose of 2 or 5 Gy.

Preparation of CD8 T cells

CD8 T cells from the spleens and lymph nodes of C57BL/6 or OT-1 TCR-transgenic mice were enriched by passing through nylon wool and treating with anti-CD4 (RL172.4), and anti-heat-stable Ag (J11D), anti-class II MHC (D3.137, M5114, CA4) mAbs, and complement. Small resting CD8 T cells were harvested from the bottom interface of a four-layer Percoll gradient (Sigma). The freshly isolated T cell populations were 90–95% CD8⁺V α 2⁺ T cells. CD8 T cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with penicillin, streptomycin, glutamine (PSG), 2-ME, HEPES, and 10% FCS (HyClone Laboratories, Logan, UT).

Preparation of Tc1 and Tc2 effector cells

For effector generation, CD8 T cells from the OT-1 transgenic mice (2×10^5 cells/ml) were stimulated with plate-bound anti-CD3 plus anti-CD28 in the presence of IL-2 (20 U/ml, supernatant from the X63Ag.IL-2 murine cell line), IL-12 (9.2 U/ml, kindly provided by Dr. Stanley Wolf, Genetics Institute, Cambridge, MA), and anti-IL-4 mAb (10 μ g/ml, 11B11) for Tc1 cultures, and in the presence of IL-2 (20 U/ml), IL-4 (200 U/ml, X63.Ag.IL-4 supernatant), and anti-IFN- γ mAb (XMG1.2, 20 μ g/ml) for Tc2 cultures. On day 4 of culture, effectors were 99% CD8⁺V α 2⁺.

Adoptive transfer

A total of 10^7 naive CD8 T cells from OT-1 mice was injected i.v. into C57BL/6 recipients 1 day after irradiation.

Analysis of cytokine production

Enriched CD8 T cells (2×10^5 cells/ml) were stimulated with plate-bound anti-CD3 (at 10 μ g/ml), IFN- γ , IL-4, and IL-5 were measured by specific ELISAs, as described previously (22).

Results

The effect of continuous BrdU exposure on T and B lymphocyte populations in unirradiated mice

It had been previously reported (3) that C57BL/6 mice die eventually if BrdU is administered daily. Ten 6-wk-old female C57BL/6 mice were divided into two equal groups. One group was placed on drinking water containing 1 mg/ml BrdU prepared fresh daily, and the other group served as the non-BrdU control. The mice were weighed before the start of the experiment and at weekly intervals, for 9 wk. It can be seen (Fig. 1) that the control group gained weight progressively, as expected, while the BrdU group languished and declined slightly in weight throughout the 9-wk period. We made no attempt to determine how BrdU admin-

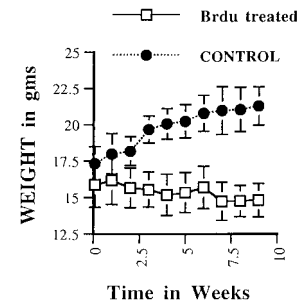


FIGURE 1. The effect of BrdU administration on weight gain in young mice. Five 6-wk-old C57BL/6 mice were placed on BrdU in the drinking water at 1 mg/ml, continuously for 9 wk, and the weight recorded each week (mean and SD). Five control mice were given drinking water without BrdU and their weight was similarly monitored.

istration limited weight gain. The mice were sacrificed at wk 9, and spleen cell suspensions were prepared. The number of spleen cells recovered from mice in the BrdU-exposed group was $6.65 \pm 1.1 \times 10^7$, approximately half that from the control group, which was $12.5 \pm 2.3 \times 10^7$. In a second experiment, the effect was even more marked with $3.1 \times 10^7 \pm 0.75 \times 10^7$ cells from the treated mice and $13.25 \times 10^7 \pm 2 \times 10^7$ in the controls.

The spleen cell suspensions were stained with a panel of fluorescent-labeled Abs and analyzed by flow cytometry. There were no obvious differences in the percentage of CD4-, CD8-, and CD19-positive cells when cells from control and BrdU-exposed mice were compared (data not shown). The level of BrdU label in each group of lymphocytes was between 33 and 50% for each category of lymphocyte. We presume that many of the cells were naive and did not divide.

Samples of the two spleen cell suspensions were adjusted to standard numbers and stimulated with plate-bound anti-CD3 and anti-CD28 for 64 h, and the thymidine uptake was determined in the last 16 h. There was no significant difference in the thymidine uptake between cells from control mice (4439 ± 1221 cpm) and BrdU-exposed mice (4426 ± 846 cpm).

The effect of continuous exposure to BrdU on the recovery of T and B lymphocyte populations from whole body irradiation

Next, we focused on cells of the immune system involved in homeostasis-driven proliferation. Mice were irradiated with 2 or 5 Gy from a cobalt source, and the number of surviving lymphocytes in the spleens of these and unirradiated mice was counted at day 1. Previous studies have shown that the number of lymphocytes falls rapidly in the period immediately after irradiation and reaches a minimum at 24 h. The fall in the log of the numbers of CD4 T cells, CD8 T cells, and B cells recovered from the spleen after exposure to 2 or 5 Gy was proportional to the irradiation dose, as previously shown, and the relative fall in the numbers of CD4 T cells, CD8 T cells, and B cells was approximately equal, with 1–5% survivors after 5 Gy, as seen by previous investigators (23). Recovery in the number of cells took place progressively in the absence of BrdU after 2 or 5 Gy (data not shown), and at an accelerating rate. The recovery of CD4 cells was the most marked, and there were $2.75\times$ as many CD4 cells at day 28 as before the irradiation. The recovery of CD8 T cells was less dramatic, reaching $2.1\times$ the preirradiation value, and B cells reached only $1.75\times$ during the period of observation.

The recovery of the mice that received 5 Gy was delayed when mice were placed on drinking water with 1 mg/ml BrdU throughout the entire period of the recovery (Fig. 2). The total cell numbers were only 75% of the preirradiation number by day 28 in mice

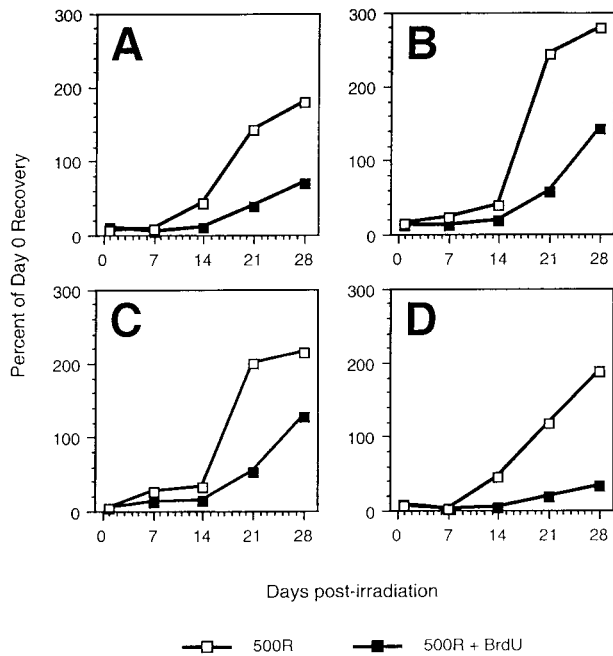


FIGURE 2. The effect of BrdU administration on lymphocyte recovery after irradiation. A group of mice was irradiated with 5 Gy whole body irradiation and placed on BrdU in the drinking water at 1 mg/ml for the entire time of the experiment. Mice were sacrificed at 1, 7, 14, 21, and 28 days, and the numbers of each cell type were determined as before. *A*, Shows total splenocytes; *B*, shows CD4 T cells; *C*, shows CD8 T cells; and *D*, shows B cells. The data points represent the mean of five mice. SDs (not shown) were within 20% of the mean.

administered BRDU (Fig. 2*A*). CD4 cells recovered more strongly at 140% at day 28, but still only half of that seen in the controls that were not exposed to BrdU (Fig. 2*B*). Comparable results were seen with CD8 T cells (Fig. 2*C*), and B cells recovered less well (Fig. 2*D*). It should be noted that in the case of the CD4 and CD8 T cells, recovery appeared to be delayed and the cells did increase in number at the same accelerating rates at the later time points that were seen earlier in the controls.

The level of BrdU label in lymphocytes that develop in the delayed recovery of irradiated mice subject to continuous BrdU exposure

Mice were irradiated with 5 Gy and allowed to recover in the presence or absence of BrdU, as before. Mice were sacrificed at days 3, 7, 12, and 20, and spleen cell suspensions were prepared and stained with Abs to CD4, CD8, CD19, CD44, and BrdU. Cells generated during the recovery in the presence of BrdU showed a progressive increase in the level of BrdU incorporation (data not shown), and they were not widely different in each of the three lymphocyte populations by day 20 (Fig. 3). There were no clear-cut differences between CD44^{low} and CD44^{high} populations (data not shown). Again, a significant number of CD4-, CD8-, and CD19-positive cells appeared to be unlabeled, even after 20 days of BrdU.

The effect of BrdU on the expansion of naive CD8 T cells adoptively transferred into irradiated recipients and challenged with Ag

We next examined the effect of BrdU on the Ag-driven proliferative response of naive cells transferred into irradiated recipients and immediately challenged with Ag. A total of 10^7 naive CD8

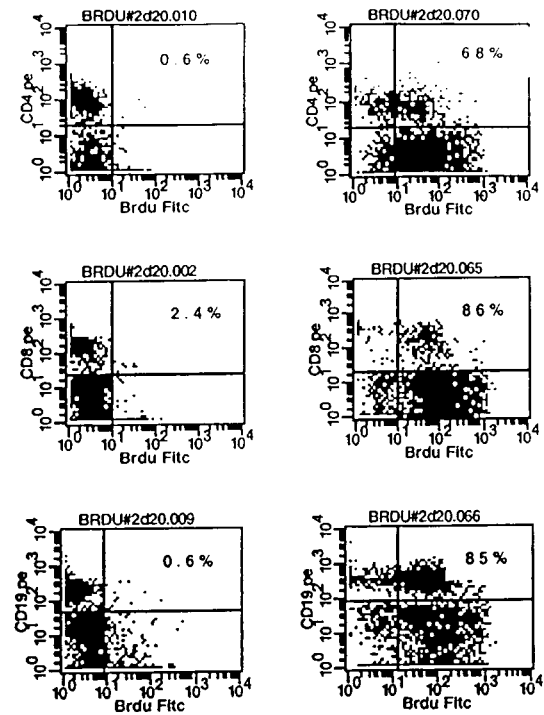


FIGURE 3. Incorporation of BrdU into recovering cells of irradiated mice. Spleen cells from a pool of five mice, sacrificed at day 20 in experiment similar in design to that described in Fig. 2, were permeabilized and stained with fluorescent-labeled Abs to BrdU, as described in *Materials and Methods*. They were then stained with fluorescent-labeled Abs to CD19, CD4, and CD8, and analyzed by flow cytometry, and the total number of cells in each lineage was determined by the product of the total cell number and the percentage in each category. *Left three panels*, Cells from recovering controls without BrdU; *right three panels*, from mice administered BrdU. *Top line*, CD4 T cells; *middle line*, CD8 T cells; *bottom line*, B cells.

cells from V α 2-positive OT-I mice was injected in each of 10 normal syngeneic C57BL/6 mice that received 5 Gy 1 day previously. BrdU was administered to five of the mice, and five served as controls. The mice were challenged by i.p. injection of 50 μ g of OVA in CFA, and the mice were sacrificed at day 7. The numbers of donor cells were determined by multiplying the total spleen cells count by the percentage of V α 2-positive, CD8-positive T cells. It can be seen that mice on BrdU generated less than half the number of V α 2 cells seen in the control by day 7 (Fig. 4). About 80% of the V α 2-positive cells were labeled at day 7, and 90% in a separate cohort run until day 14 (data not shown).

The function of BrdU-labeled T cells

Naive CD8 T cells from OT-I mice were transferred into irradiated recipients in the same experiment as above. CD8 cells were prepared from the same 7-day spleens, adjusted to standard cell numbers, and challenged in vitro with 3-day B cell blasts pulsed with the OVA peptide. Supernatants were harvested at 24 and 48 h and were assayed by ELISA for IFN- γ , IL-4, and IL-5 to see whether the effectors from BrdU-treated mice make the same amount of cytokines per cell as those from the control mice. It can be seen (Fig. 5) that the cells from BrdU-treated mice were less effective per comparable number of cells in the secretion of IFN- γ upon restimulation and make less than half the amount of cytokine made by the same number of cells from the control mice. No IL-4 or IL-5 was secreted by either set of cells (data not shown).

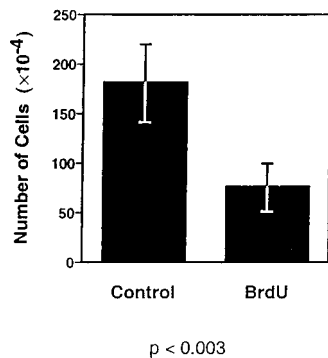


FIGURE 4. The effect of BrdU administration on cell responding to Ag. A total of 10^7 naive $V\alpha 2^+$ cells from young OT-1 TCR transgenic mice was injected into each of 10 syngeneic C57BL/6 hosts that had been irradiated with 5 Gy on day -1 and was injected with $50 \mu\text{g}$ OVA i.v. on day 0. Half of the mice were placed on BrdU in the drinking water at 1 mg/ml, and the other half served as controls. Mice were sacrificed at day 7, spleen cell suspensions were prepared and counted, and the cells were stained with fluorescent-labeled Abs to $V\alpha 2$ and to CD8 and analyzed by flow cytometry. The total number of $V\alpha 2^+$, CD8 $^+$ responding cells was determined for each mouse, and the average for the BrdU and control mice was determined. The data represent the mean and SD of readings from five individual mice per group. The p value was determined by the Student t test.

Effect of BrdU on CD8 T cells in vitro

Previous studies (13) conducted many years ago had shown that low concentrations of BrdU inhibited the development of Ab formation in cultures of rabbit spleen cell suspensions from rabbits that were stimulated with Ag in vivo 2 days before culture. It was concluded, at the time, that the BrdU inhibited the expansion of the population of Ab-forming cells. The design of this experiment was archaic, and a somewhat different protocol was adopted in this study. CD8-enriched populations from the spleens of OT-1 TCR transgenic mice were stimulated in vitro with plate-bound anti-CD3 and anti-CD28 for 4 days in the presence of varying concentrations of BrdU. The cells were cultured under one of two conditions to polarize the response with respect to cytokine secretion (24). One group was stimulated in the presence of IL-12 and anti-IL-4 to produce Tc1 effectors, while the other group was stimulated with IL-4 and anti-IFN- γ to generate Tc2 effectors. The total number of cells generated in the presence of BrdU was reduced (Fig. 6).

The effector populations were adjusted to standard cell numbers and restimulated with plate-bound anti-CD3, and the production of cytokines in the 24- and 48-h supernatants was determined by

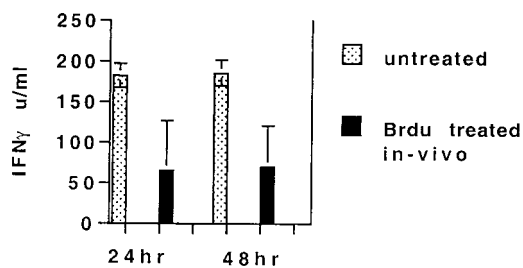


FIGURE 5. The effect of BrdU incorporation on T cell function. Pools of spleen cell suspensions from the same five control and five BrdU mice sacrificed at day 7 in the experiment described in Fig. 4 were adjusted to standard cell numbers and challenged in vitro with B cells pulsed with the OVA peptide SIINFEKL. Culture supernatants were harvested at 24 and 48 h and analyzed for IFN- γ , IL-4, and IL-5 by ELISA. No IL-4 or IL-5 was seen. The data represent the mean and SD of values for triplicate cultures.

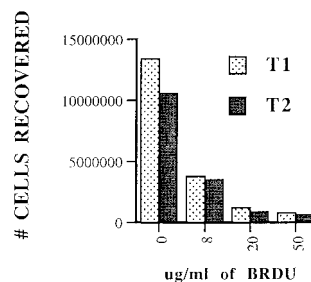


FIGURE 6. CD8-enriched populations of spleen cells from OT-1 mice were prepared and cultured on plate-bound anti-CD3 and anti-CD28 for 4 days in the presence of various concentrations of BrdU. Tc1 cells (T1) were generated in the presence of IL-2 and IL-12, while Tc2 cells (T2) were generated in the presence of IL-2 and IL-4. Cells were harvested at day, and the total number of live cells was determined for each concentration of BrdU and for each T cell subset. The data represent the mean and SD of values for triplicate cultures.

ELISA. Once again, cells that had been grown in the presence of BrdU made smaller amounts of either Tc1 or Tc2 cytokines, as shown in Fig. 7.

Discussion

The experiments outlined above show that cells that proliferate in mice who are exposed to BrdU in the drinking water under the standard conditions used to label dividing cells perform less well than cells from control mice in several experimental models.

In the first model, we examined the gross effect of continuous exposure to BrdU in young female mice 6 wk old at the start of the experiment. Those fed BrdU failed to gain weight and were also sickly in appearance. We made no attempt to determine which cells were involved in the arrest of normal weight gain, but presume multiple mechanisms may be involved. We did, however, examine whether there were any detectable departures from the normal in the relative numbers of T and B lymphocyte populations, and none were discerned. The mice were sacrificed after 9 wk of exposure, and cells were also stained with Abs to BrdU, and moderate levels of BrdU incorporation were revealed. We made no measurement of incorporation at intermediate times in this experiment, but saw somewhat higher levels of label in the recovery from irradiation model (68–86% seen in Fig. 3) and the adoptive transfer model (80–90%) in which earlier measurements were made. It should be noted, however, that the three models differ also with respect to the stimulus to division. In the long-term exposure experiment, there was no additional stimulus to divide other than the normal turnover and overall growth of the animals, while in the

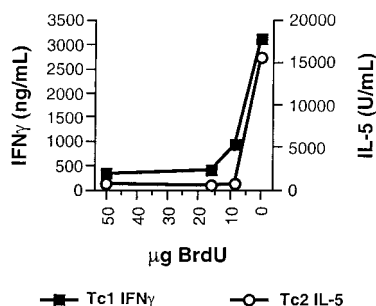


FIGURE 7. CD8 T cells from the experiment described in Fig. 6 were restimulated in vitro, as described in this figure, and the IFN- γ and IL-5 were determined in the culture supernatants of the Tc1 and Tc2 effectors, respectively. The data represent the mean and SD of values for triplicate cultures.

recovery from irradiation model, cells were responding to homeostasis signals, driving the restoration of normal lymphocyte numbers after irradiation, and in the adoptive transfer model the proliferation of all the $V\alpha 2$ cells was Ag driven. When the cells from the long-term BrdU-fed mice were stimulated in vitro with anti-CD3 and anti-CD28, they showed the same rate of thymidine incorporation as did cells from the control mice.

It is possible that the cells surviving in mice exposed to BrdU for a very long time were selected for those that had lost the salvage pathway and were no longer able to incorporate deoxynucleotides. Our experiments did not allow us to determine whether such selection had occurred or whether cells already labeled with BrdU were able to incorporate thymidine.

In the second model, we looked at the recovery of lymphocyte populations after whole body irradiation. It is believed that a substantial part of the recovery comes from the division of lymphocytes that survived irradiation, while other cells are regenerated from surviving stem cells. The lymphocyte populations in the normal mice expanded ~100-fold during the recovery period and must have undergone a minimum of seven rounds of division, and much more if there was significant cell death or other heterogeneity in the process. In either event, the recovery of lymphocyte numbers after irradiation occurs more slowly in mice exposed to BrdU than do those from control mice, and the recovery is delayed, as shown in Fig. 2. This is true for CD4-, CD8-, and CD19-positive lymphocytes, and there are no obvious differences between the effect on T cells and B cells. It would appear that after the initial lag in recovery, the expansion of the CD4- and CD8-dividing cells proceeds at the same rate as in the control, but this was not seen for B cells. It is possible that this later growth is due to cells that have reduced levels of BrdU incorporation due to changes in the relative use of the de novo and salvage pathways for the synthesis of 5 methyl deoxyuridine monophosphate. The fact that a substantial fraction of the expanded lymphocyte population is still unlabeled with BrdU even after 20 days exposure to BrdU in the drinking water (shown in Fig. 3) is compatible with this possibility.

In the third model, we looked at the effect of BrdU on the response of naive CD8 T cells to Ag. In this study, we used naive cells from OT-1 TCR transgenic mice adoptively transferred into irradiated mice, which were then challenged with OVA. In this model, the cells that we followed were not themselves irradiated in contrast to the cells in the previous model. Again, the expansion was reduced by the incorporation of BrdU (Fig. 4), showing that the effect was not due to a combination of irradiation and BrdU, but to BrdU alone. In addition, the labeled cells were shown to be less able to secrete cytokines when challenged in vitro, as shown in Fig. 5. Whether this was due to a higher incidence of cell death in vitro challenge or to some other effect was not determined.

Finally, we reexamined the effect of BrdU in vitro. In this study, it was clear that BrdU severely inhibited cell growth and again led to a compromised ability of the Tc1 and Tc2 effectors to make cytokines.

It is clear that cells that have proliferated in the presence of BrdU, under the conditions commonly used to enumerate dividing cells, are less able to undergo further division and are less able to perform effector functions. This is not likely to affect the validity of the interpretations drawn in previous studies, if BrdU is used only to determine the number of cells in cycle over a brief labeling period.

The deleterious effects observed in inhibition of weight gain or homeostatic regeneration after exposure to irradiation were the result of several weeks of exposure to BrdU. In the studies in which we examined the effect of Ag-driven expansion in vivo, however, the time was only 7 days, while the in vitro studies lasted only 4 days. The effect of BrdU in vitro appears to be much more severe than in vivo and may not be relevant to in vivo models.

In studies in which cells are labeled for 7 days or more in vivo, it is likely that the subsequent performance of such cells is compromised, both in their potential for further division and for carrying out their effector function. Alternatively, it may be that the cells that do function in the mouse after prolonged exposure to BrdU represent a subset that either was or became resistant to BrdU as a result of changes in the relative use of the de novo and salvage pathways for nucleotide synthesis. This may or may not affect the validity of the interpretation of such experiments, according to the nature of the conclusions drawn.

Acknowledgments

We are indebted to Gregory Building Supply (Ray Brook, NY) for support for the summer High School Intern scholarship for D.S.J.

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